*Original research paper

Antibacterial and antiviral activities of essential oils of northern Moroccan plants

Naima EL MOUSSAOUI[a*], Gloria SANCHEZ[b], El Ouardy KHAY[a], Mohammed IDAOMAR[a], Ahmed IBN MANSOUR[c], Jamal ABRINI[a], Rosa AZNAR[b,d]

[a] Laboratory of Biology and Health, Department of Biology, Faculty of Sciences, University Abdelmalek Essaâdi, M'hannech II, BP 2121, Tetouan 93002, Morocco.

[b] Institute of Agrochemistry and Food Technology (IATA). Spanish Council for Scientific Research (CSIC), Spain.

[c] Applied organic chemistry laboratory, Department of Chemistry, Faculty of Sciences, University Abdelmalek Essaâdi, M'hannech II, BP 2121, Tetouan 93002, Morocco.

[d] Department of Microbiology, Faculty of Biological Sciences, University of Valencia, Av Dr Moliner 50, E-46100 Burjassot, Valencia, Spain.

Abstract

Aims: This study was designed to evaluate the antimicrobial activity of five essential oils (EOs) extracted from the aerial parts (leaves and flower summits) of three species growing in the north of Morocco: *Origanum elongatum*, *Thymus capitatus* and *Mentha suaveolens*.

Place and Duration of Study: Department of Biology (Faculty of Sciences), Institute of Agrochemistry and Food Technology (IATA), between September 2009 and December 2009.

[*] *Corresponding author: Email: nmelmoussaoui@gmail.com*

Laboratory of Biology and Health, Department of Biology, Faculty of Sciences, University Abdelmalek Essaâdi, M'hannech II, BP 2121, Tetouan 93002, Morocco

Methodology: The EO constituents were extracted by hydrodistillation and analysed by GC-MS. The antibacterial activity of the essential oils was tested against *Salmonella*, *Listeria monocytogenes* and *Escherichia coli* O157:H7, using the diffusion method and the microtitration assays. The antiviral effect of EOs was evaluated for the inactivation of murine norovirus (MNV-1), a human norovirus surrogate.

Results: The most important compounds were carvacrol, thymol, *p*-cymene and piperitenone oxide. All extracts exhibited an antibacterial activity at different levels against strains reported as the causal agents of foodborne diseases but a low antiviral activity (0.87-0.50 $\log_{10}$ $TCID_{50}$/ml reduction) was observed.

Conclusion: Results suggest the potential use of tested EOs as bio-preservative in the food industry. However, their antiviral activity needs to be further investigated.

***Keywords**: Origanum elongatum*; *Thymus capitatus*; *Mentha suaveolens*; Essential oil; Antibacterial and antiviral activity.

1. Introduction

Since society is experiencing a trend toward 'green consumerism', with a desire of fewer synthetic food additives and products with a smaller impact on the environment, the use of naturally derived antimicrobials has substantially increased in the last decade.

In this sense, biopreservatives are a wide range of natural products that can be used to reduce or eliminate pathogen populations while increasing food quality. Among this type of antimicrobials, essential oils (EOs) have long been applied as flavoring agents in food, and due to their content in antimicrobial compounds, they have potential as natural agents for food preservation [1].

Essential oils or their main active compounds have been reported to possess a wide spectrum of antibacterial [2, 3], antiviral [4, 5], antifungal [6], antioxidant [7], antiparasitic [8], insecticidal [9] and cytotoxic properties [10].

Essential oils are the volatile oily liquids of the secondary metabolism of scented plants, which are obtained from different plant parts, such as flowers, leaves, seeds, bark, fruits and roots. They are also widely used as food flavours and preservatives to prevent growth of food-borne bacteria and molds, and so extend the shelf life of processed foods [1]. Recently, the antiviral effects of different EOs have been evaluated on human norovirus surrogates [11, 12].

We report here the antimicrobial properties and the chemical composition of essential oils of three plants (leaves and flower summits). The antibacterial activity of EOs was evaluated on *Salmonella*, *Listeria monocytogenes* and *Escherichia coli* O157:H7, determining the zone of inhibition growth, minimum inhibitory and bactericidal concentrations (MIC and MBC). The antiviral activity was tested on the murine norovirus (MNV-1) a human norovirus surrogate by the 50% tissue culture infectious dose ($TCID_{50}$) in RAW 264.7 cells.

2. Materials and Methods

2.1. Plant materials and Hydrodistillation

Three plants were collected during the April- august period of 2009 in the areas around Tetouan, and Al-Housaima (northern Morocco). They were identified by Prof. M. Kadiri of Department of Biology, Faculty of sciences (Tetouan, Morocco) as *Thymus capitatus* (L.) Hoffmanns. & Link, (leaves, flower summits), *Origanum elongatum* (Bonnet) Emb. & Maire (leaves, flower summits) and *Mentha suaveolens* Ehrh. (leaves).

Aerial parts of collected plants were air-dried at room temperature and stored in a dry place prior to use, then grossly pulverized and subjected to hydrodistillation for 3 h using a Clevenger apparatus.

2.2. Essential oils chemical analysis

The chemical compositions of essentials oils were analyzed using a gas chromatograph (*TRACE GC Ultra*) fitted to a mass spectrometer (*Polaris Q-Ion Trap MS*). Operating in electron-impact EI (70 eV) mode. VB-5 (Methylpolysiloxane 5% phenyl) and a column (30 m × 0.25 mm × 0.25 µm thickness) were used (*Centre National pour la Recherche Scientifique et Technique- CNRST*, Rabat, Morocco). The chromatographic conditions were as follows: Injector and detector temperatures at 220 and 300°C, respectively; carrier gas, helium at flow rate of 1.4 ml/min; temperature program ramp from 40 to 300°C with gradient of 4°C/min (holding the initial and final temperature for 4 min). The relative amount of individual components of the total oil was expressed as a percentage peak area relative to total peak area. Library search was carried out using the combination of NIST MS Search and a personnel aromatic library.

2.3. Bacterial strains

Experiments were performed on three reference strains supplied by the *Spanish Type Culture Collection* (CECT): *Salmonella enterica* subsp. *enterica* CECT 915^{T}, *Listeria*

monocytogenes CECT 4031^T, and *Escherichia coli* O157:H7 CECT 4267, and two food isolates supplied by the Public Health Service of Valencia (Spain), one *Salmonella* sp isolated from mayonnaise and one *Listeria monocytogenes* from seafood salad.

Stock bacterial inoculum suspensions were obtained from 18 h culture in Luria Bertoni broth (LB) at 37°C. After, the strains were re-inoculated approximately for three hours for *Escherichia coli* and *Salmonella*, and five hours for *Listeria*. Those final suspensions served for the inoculum preparation. The cell density of each suspension was determined spectrophotometrically, and then adjusted to a concentration of 10^6 CFU/ml.

2.4. Virus and cells culture

The cytopathogenic murine norovirus (MNV-1) (kindly provided by Prof. H. W. Virgin, Washington University School of Medicine, USA) was propagated and assayed in RAW 264.7 cells. Semi-purified stocks were subsequently produced from the same cells by centrifugation of infected cell lysates at 660 × g for 30 min. Infectious viruses were enumerated by determining the 50% tissue culture infectious dose ($TCID_{50}$) with eight wells per dilution and 20 µl of inoculum per well.

RAW 264.7 cell lines were maintained in Dulbecco's Minimum Essential Medium (DMEM, HyClone), supplemented with 10% of fetal bovine serum (FBS, HyClone), 100 U/ml penicillin, 100 µg/ml streptomycin (HyClone), 10 mM hydroxyethyl piperazine ethane sulphonic acid buffer (HEPES, HyClone) and 2 mM L-glutamine (HyClone). Cell lines were grown in humidified atmosphere of 5% carbon dioxide at 37°C.

2.5. Antibacterial activity tests

2.5.1. Agar diffusion method

The disk-diffusion assay was used to test antibacterial activity of the essential oils against five bacteria according to Bauer et al. [13]. Sterile filter papers disks (6 mm in diameter) impregnated with essential oils were placed on nutrient agar medium uniformly

seeded with a broth culture (10^6 CFU/ml) of the test microorganisms. These plates were then kept at low temperature (4ºC) for 2 h to allow maximum diffusion, and incubated at 37ºC for 24 h. The diameter in millimeter of the inhibition zones around the disks was recorded. All the tests were performed in triplicate.

2.5.2. Microtitration method

Essential oils were diluted in Lysogeny broth (LB) supplemented with bacteriological agar 0.15% (w/v). Serial twofold dilutions, ranging from 2 % (v/v) of essential oil, were prepared in a 96-well microtitre plate, volume being 50 µl. Wells were then inoculated with 50 µl of microbial suspension at final concentration of 10^6 CFU/ml. The covered microplates were incubated overnight at 37°C. To assess microbial growth, 10 µl of resazurin were added to the wells. After incubation at 37°C for 2 h, the minimum inhibitory concentration (MIC) was then determined as the lowest essential oil concentration prevented change of coloring of resazurin [14]. The minimum bactericidal concentration (MBC) corresponded to the lowest concentration of the essential oils yielding negative subcultures after incubation at appropriate temperature for 24 h. It was determined in broth dilution tests by subculturing 10 µl from negative wells on PCA medium [14].

2.6. Antiviral activity assays

EOs at 2% were added to MNV suspensions in DMEM with 2% FCS and further incubated at 37°C for 1 h. Then, ten-fold serial dilutions of mixture prepared were re-incubated for 1 h. After that, RAW 264.7 cells were inoculated with serial dilutions of MNV-1 and EOs mixture from 10^0 to 10^{-8}. Positive controls were MNV suspensions without EO. Then, infectious viruses were enumerated by cell-culture assays as described above. Virucidal activity of EOs was estimated by comparing the number of infectious viruses on suspensions without EOs and on the EO-treated virus suspensions.

3. Results and discussion

3.1. Chemical composition of essential oils

Essential oils were isolated by hydrodistillation from the aerial parts of *Thymus capitatus* (leaves, flower summits), *Origanum elongatum* (leaves, flower summits) and *Mentha suaveolens* (leaves). The mains components are summarized in Table 1.

Thymus capitatus essential oil was mainly constituted by carvacrol (58.77-68.63%), along with other components to relatively low levels of *p*-cymene (4.84-5.63%), γ-terpinene (2.78-3.75%) and β-caryophyllene (2.62-2.91%). It was substantially similar to that of EOs originating in Tunisia collected during different phases of plant development, and from different locations [15]. Many authors have found that carvacrol is the main component of thyme EOs: El Ajjouri et al. [16] (70.92%); Faleiro et al. [17] (79%); Hedhili et al. [18] (53.71%); Bouzouita et al. [2] (62-83%); Benjilali et al. [19] (78%). Similarly, Turkish thyme EO was dominated by only 35.6% of carvacrol followed by 18.6% of thymol [20]. However, the main constituents of thyme EO from Sardinia are thymol (29.3%) and *p*-cymene (26.4%), while, carvacrol represents only 10.8% of the species [21].

Besides, the main components of oregano EO were carvacrol (19.21-40.12%), thymol (3.57-14.24%), *p*-cymene (16.08-16.19%) and γ-terpinene (7.27-13.48%). Benjilali et al. [22] and Figueredo et al. [23] had characterized a higher quantity of carvacrol (36.6-76.6%) and (62.8-79.2%), respectively. While Velasco-Negueruela et al. [24] had characterized carvacrol (24.5-51.6%). In addition, the two components *p*-cymene (16.08%) and γ-terpinene (7.27%) of oregano EO obtained from flower summits was near to those analyzed by Figueredo et al. [23]. Also, the EO obtained from leaves differed appreciably with non-negligible levels of thymol (14.24%) like it founded by Figueredo et al. [23] with two samples (13.6% and 17.2%). These analyzes indicate that thyme and oregano EOs are not always dominated by carvacrol.

On the other hand, mint EO was characterized by piperitenone oxide (41.84%), (-)-isopulegol (11.95%) and limonene (7.35%). In fact, in most cases, the large majority constituents of mint EOs were menthanic oxygenated monoterpenes [6, 25, 26, 27, 28, 29, 30, 31]. Besides, Oumzil et al. [6] eminent three profiles of mint EOs depending on the subspecies. One of these profiles was dominated by piperitenone oxide (56%) as our oil tested. The same was for mint EO from Japan (87.3%) [25].

3.2. Antibacterial activity

Initial screening of the antibacterial activity of the investigated EOs was studied against five tested microorganisms using the agar disk diffusion assay. The antibacterial activity of EOs can be classified into three levels [32, 33]: (i) weak activity (inhibition zone≤12 mm), (ii) moderate activity (12 mm<inhibition zone<20 mm) and (iii) strong activity (inhibition zone≥20 mm). Results from the agar disk diffusion tests for antimicrobial activity of the EOs are shown in Table 2.

The investigated EOs had a variable degree of activity against different tested strains. The oregano and thyme EOs showed the highest activity against all the tested microorganisms, especially for Gram-positive bacteria (*Listeria*) and Gram-negative bacteria (*Salmonella*), whose zones of inhibition ranged from 21.7 mm to 34.3 mm. While, a moderate activity was observed against *E. coli*, with zones of inhibition ranged from 14.3 mm to 19.7 mm. Rather, the mint EO showed weak activity against *E. coli* and *Listeria*. However, moderate activity was observed against *Salmonella*.

Generally, Gram-negative bacteria are slightly less susceptible to the presence of EOs from spices and herbs than Gram-positive bacteria [3]. This can be referred to the outer membrane surrounding the cell wall of Gram-negative bacteria, which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering [34]. In contrast, in this

study, the Gram-negative bacteria *Salmonella* was found to be most susceptible to oregano and thyme EOs as for Gram-positive bacteria *Listeria*.

Furthermore, the microtitration assays were attributed to determine the MICs and MBCs of the tested EOs. The MICs and MBCs of the EOs against the tested strains are presented in Table 3 and 4. The MICs and MBCs values confirmed the results obtained by the agar disk diffusion method. Oregano and thyme EOs had the lowest MICs (0.0625-0,125 %) and MBCs (0.125-0.25%) against *Salmonella*, while *Listeria* was inhibited with MICs ranged from 0.0625% to 0.5% and MBCs varied between 0.25% and 1%. Moreover, the mint EO was less active against all the tested microorganisms with MICs and MBCs between 0.5% and >2%. Besides, the tested oils inhibited *E. coli* at MICs and MBCs ranged from 0.125 to 0.5%.

However, comparing the two assays, the oils exhibited a variation on the inhibition of the growth of *E. coli* and *Listeria* L23. In fact, we found that *E. coli* resisted more the oils tested in agar medium than *Listeria* L23, while, in microtitration assays, *Listeria* L23 was more resistant. This difference in activity can be explained by the oil solubility and volatility as reported by Bouhdid et al. [14] and Hernandez et al. [35].

In this study, thyme and oregano EOs showed its best activity as well against Gram-negative and Gram-positive bacteria. Similar results were reported previously for thyme and oregano EOs obtained from different species [2, 14, 17, 36, 37, 38, 39]. While, Bounatirou et al. [15] founded that thyme EO had a low activity against *Salmonella* strain. Besides, mint EO (*M. suaveolens*) had a low activity as observed by Sutour et al. [40]

The inhibitory activity of oregano and thyme EOs is probably mainly due to the phenolic constituent (carvacrol 19.21-68.63% and thymol 14.2-43.57%). Actually, several authors have pointed to the antimicrobial activity of carvacrol and thymol against *E. coli* O157: H7 in *in-vitro* experiments [36, 41, 42]; as to its mechanism of action, this two compounds inhibited *E. coli* O157: H7 by disintegrating the outer membrane and releasing

outer membrane-associated material from the cells to the external medium as suggested by Helander et al. [43]. Moreover, Gill and Holley [44] have found that carvacrol inhibited *E. coli* and *L. monocytogenes* motility, by inhibiting ATPase activity and disrupting the membrane.

Although, comparing oregano and thyme EOs from leaves and flower summits of the same plant, they showed a slight difference in either minimal inhibitory or bactericidal concentrations, while, the percentage of the mains components (carvacrol, thymol and γ-terpinene) varied for oregano EOs, as it was observed by Peñalver et al. [45]. In fact, an additive effect between the mains components has been suggested [14, 42].

On the other hand, mint EO was mainly composed by peperitenon oxide which was considerate to have a low antimicrobial activity due to the presence of an epoxide between C1 and C2 [6]. However, mint EO showed the same minimum bactericidal concentration as oregano EO against *E. coli* (E3). This result pointed especially the important role of minor components [7, 46].

Actually, components, such as γ-terpinene, *p*-cymene and limonene, have been considered to display relatively good activity [46]. Indeed, γ-terpinene did not antagonize the growth of *E. coli* O157:H7 [42] and *S. typhimurium* [47]. Also, *p*-cymene showed no antibacterial activity against *E. coli* O157:H7 in *in-vitro* experiments [42], while, it was inhibited in *in-vitro* [48] and in apple juice [49]. As well, limonene had moderate antimicrobial activity against *E. coli* O157:H7 [6] and low antilisterial properties [50]. Besides, Dorman and Deans [51] observed that limonene was more active than *p*-cymene.

Moreover, Cristani et al. [48] suggested that the antimicrobial effect of carvacrol, thymol, γ-terpinene and *p*-cymene may result, from a gross perturbation of the lipidic fraction of the plasmic membrane of the microorganism. In addition, the biological precursor of carvacrol, *p*-cymene causes swelling of the cytoplasmic membrane to a greater extent than

does carvacrol [52]. *p*-Cymene when combined with carvacrol a synergism has been observed against *B. cereus* in vitro and in rice [53].

3.3. Antiviral activity

In this study, the results obtained showed that the EOs tested at 2% had no or low antiviral activity against MNV-1 (Table 5). Mint EO represented a reduction in viral titer of 0.87 $\log_{10}$ $TCID_{50}$/ml. This was followed by flower summits of oregano and thyme EOs with 0.75 and 0.5 $\log_{10}$ $TCID_{50}$/ml reductions, respectively.

However, Elizaquível et al. [12] founded that oregano EO decreased MNV titers by 1.04-1.62 $\log_{10}$ $TCID_{50}$/ml and clove EO showed reductions of 0.67 $\log_{10}$ $TCID_{50}$/ml at 37°C while zataria EO showed no notable reductions in MNV titers. Also, Kovač et al. [11] observed no reduction of norovirus surrogates titers by using hyssop and marjoram EOs at different temperatures and times.

On the other hand, Lee et al. [54] founded that pretreated cells with red ginseng extract or ginsenosides reduced the titer of MNV between 0.37-1.48 $\log_{10}$ $TCID_{50}$/ml depending on the virus concentration while co-treatment or post-treatment were not effective.

In addition, it has been reported that chitosan [55], grape seed extract [56], pomegranate juice, pomegranate polyphenols [57, 58], cranberry juice and cranberry proanthocyanidins [59, 60]. reduce to some extend the titer of foodborne viral surrogates. For MNV, the highest reduction was observed by cranberry proanthocyanidins with almost 3 log reduction [59].

In fact, non-enveloped viruses are less susceptible to EOs, due to the lack of the lipid envelope, for example: adenovirus was not affected by eucalyptus EO [4], the same for poliovirus 1 and adenovirus 2 treated with tea tree EO [61].

Behravan et al. [5] suggest that antiviral activities of *Thymus transcaspicus* EO against *Bacillus* phage CP51 were due to the presence of carvacrol and thymol. In addition, Lai et al. [62] founded that herpes simplex virus type I HSV-1, an enveloped DNA virus, was 90 %

inactivated directly within 1 hour by both carvacrol and thymol. Also, the human rotavirus (RV), a non-enveloped virus, was not inhibited by the Mexican oregano EO, while carvacrol alone exhibited high antiviral activity [63].

However, it has been reported the inactivation of HSV-1 particles viral infection by monoterpene compounds such as γ-terpinene, *p*-cymene and thymol from thyme oil [64]. In contrast, Garozzo et al. [61] founded that compounds α-terpinene, γ-terpinene and *p*-cymene were ineffective against ECHO 9, Coxsackie B1, HSV-1 and HSV-2 viruses.

The sesquiterpene hydrocarbon β-caryophyllene, present in all tested EOs, has been reported as the most active antiviral compound on HSV. However, their antiviral effects decrease moderately by the introduction of either an epoxide or hydroxyl functions into this backbone [65].

Besides, Korean red ginseng extract reduced the viral infectivity of norovirus surrogates through an anti-adhesive effect of carbohydrates as suggested [54]. As well, high levels of polyphenols, anthocyanins, and glycosides in pomegranate extract have been proposed to be antiviral ingredients [57, 66].

In this study, MNV-1 was incubated with EOs prior to host cell infection to investigate if the EOs might interfere directly with virus capsid structures as proposed by others authors [57, 66, 67]. However, antiviral activity of tested EOs needs to be further investigated.

4. Conclusion

In conclusion, the interactions between different components of EOs play an important role on their biological activity. Based on their composition, EOs display different activity on bacteria and virus. The antibacterial and the slight antiviral activity of tested oregano, thyme and mint EOs fostered their potential as bio-preservatives to improve food safety.

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COMPETING INTERESTS

Authors declare no conflict of interest.

Authors' contributions

'Author EL MOUSSAOUI N' has performed the overall practical study, interpreted and wrote the first draft of the manuscript. 'Author SANCHEZ G' has written the protocol of the antiviral activity and read the respected outcomes. 'Author IBN MANSOUR A' has read the result of the chemical composition of essential oils. 'Author AZNAR R and ABRINI J' have written the protocol of antibacterial activity. 'Author KHAY EO' provided technical advices. All authors read and approved the final manuscript.

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Table 1 Essential oil composition identified by GC

RT (min)	Compounds	*M. suaveolens*	*O. elongatum*		*T. capitatus*	
		L	L	FS	L	FS
11.42	*p*-Cymene	0.42	16.19	16.08	4.84	5.63
11.59	Limonene	7.35				
12.68	γ-Terpinene	1.06	13.48	7.27	3.75	2.78
16.83	(-)-Isopulegol	11.95				
20.98	Thymol		14.24	3.57	0.04	0.07
21.28	Carvacrol		19.21	40.12	68.63	58.77
23.24	Piperitenone oxide	41.84				
24.93	β-Caryophyllene	1.32	1.38	0.86	2.91	2.62

L: leaves; FS: flower summits, RT: Retention time.

Table 2 Inhibition zones of essential oils against tested bacteria

		Inhibition zones diameter [a] (mm)				
		M. suaveolens	*O. elongatum*		*T. capitatus*	
		L	L	FS	L	FS
Salmonella	S36^{T}	12.7	34.3	31.5	33.7	21.7
	S64^{F}	13	28.2	30.7	29	22.3
Escherichia coli O157:H7	E3^{T}	6.2	19.7	18	14.3	18.5
Listeria monocytogenes	L3^{T}	8.3	34	29	29	28.7
	L23^{F}	3.3	31	33	25	28

[a] The diameter of the disks (Ø=6 mm) was not included; L: leaves; FS: flower summits; T: type strain; F: food isolated strain; S36: *Salmonella enterica* subsp. *enterica* CECT 915^{T}; S64: *Salmonella* sp isolated from mayonnaise; E3: *Escherichia coli* O157:H7 CECT 4267; L3: *Listeria monocytogenes* CECT 4031^{T}; L23: *L. monocytogenes* isolated from seafood salad.

Table 3 Minimal inhibitory concentration (MIC) of essential oils (v/v %) against tested bacteria

		MIC				
		M. suaveolens	*O. elongatum*		*T. capitatus*	
		L	L	FS	L	FS
Salmonella	S36[T]	0.5	0.0625	0.0625	0.0625	0.125
	S64[F]	0.5	0.0625	0.0625	0.0625	0.125
Escherichia coli O157:H7	E3[T]	0.5	0.25	0.25	0.25	0.125
Listeria monocytogenes	L3[T]	1	0.125	0.125	0.0625	0.125
	L23[F]	>2	0.5	0.5	0.5	0.5

L: leaves; FS: flower summits; T: type strain; F: food isolated strain; S36: *Salmonella enterica* subsp. *enterica* CECT 915[T]; S64: *Salmonella* sp isolated from mayonnaise; E3: *Escherichia coli* O157:H7 CECT 4267; L3: *Listeria monocytogenes* CECT 4031[T]; L23: *L. monocytogenes* isolated from seafood salad.

Table 4 Minimal bactericidal concentrations (MBC) of essential oils (v/v %) against tested bacteria

		MBC				
		M. suaveolens	*O. elongatum*		*T. capitatus*	
		L	L	FS	L	FS
Salmonella	S36[T]	1	0.125	0.125	0.125	0.125
	S64[F]	0.5	0.125	0.125	0.125	0.125
Escherichia coli O157:H7	E3[T]	0.5	0.5	0.5	0.25	0.125
Listeria monocytogenes	L3[T]	>2	0.125	0.25	0.125	0.125
	L23[F]	>2	0.5	0.5	1	0.5

L: leaves; FS: flower summits; T: type strain; F: food isolated strain; S36: *Salmonella enterica* subsp. *enterica* CECT 915[T]; S64: *Salmonella* sp isolated from mayonnaise; E3: *Escherichia coli* O157:H7 CECT 4267; L3: *Listeria monocytogenes* CECT 4031[T]; L23: *Listeria monocytogenes* isolated from seafood salad.

Table 5 Antiviral activity of EOs against MNV-1

	M. suaveolens	*O. elongatum*		*T. capitatus*	
	L	L	FS	L	FS
Control	$5.90\ 10^{7}$	$2.50\ 10^{6}$	$1.87\ 10^{7}$	$2.50\ 10^{6}$	$1.87\ 10^{7}$
EO-treated virus	$7.90\ 10^{6}$	$5.90\ 10^{6}$	$3.30\ 10^{6}$	$7.90\ 10^{6}$	$5.90\ 10^{6}$
Antiviral activity	-0.87	0.37	-0.75	0.49	-0.50

L: leaves; FS: flower summits.